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TITLE: Exploiting for Breast Cancer Control a Proposed Unified Mechanism for Reduction of Human Breast Cancer Risk by the Hormones of Pregnancy

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14. ABSTRACT Results in the third grant year further support the "Unified Mechanism Hypothesis" in that: (1) Giving pregnancy associated hormones or hCG to virgin female rats either before or after MNU treatment elicits persistent serum AFP levels, thereby explaining why breast cancer appearance is inhibited when employing either treatment sequence; (2) Giving hCG to estrogenized SCID mice bearing human breast cancer xenografts inhibits the cancer growth, apparently by action of murine AFP that the treatment has elicited from the mouse liver as an inform with low avidity for our available anti mAFP antibody. We are able to detect mAFP in mouse serum by western blot when it is present in the very high concentration that is elicited by injection of the animals with high E3 doses. (3) In an "all-human" in vitro system, hCG elicits hAFP from cultured HepG2 human liver cancer cells, addition of the hAFP-containing supernate to cultures of MCF7 human breast cancer cells blocks their growth, and that adding anti hAFP antibody to that system prevents the inhibition. hAFP is thus confirmed as the proximal inhibitor.					
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Exploiting for Breast Cancer Control a Proposed Unified Mechanism for Reduction of Human Breast Cancer Risk by the Hormones of Pregnancy.

INTRODUCTION

The substantial reduction of life-time risk for breast cancer that early term pregnancy confers on women has drawn the attention of researchers seeking new modalities for reducing the disease incidence among US women from the current level of 200,000 new cases/year. They have administered different pregnancy-associated hormones (steroid estrogens and/or progestins, or hCG) to carcinogen-treated virgin rats and have in most cases produced considerably reduced breast cancer occurrence.

We hypothesized that in each of these successful treatments the administered hormone has elicited secretion of AFP from the adult rat liver. This then appears in the serum, as it does in pregnancy, in which state AFP of fetal origin enters the maternal serum. We have shown in many studies that AFP is a potent anti-breast cancer agent. Our aims in this project have been: (Aim 1) to replicate previously published studies showing that each hormone of pregnancy is capable of reducing the incidence of carcinogen-induced breast cancer and to show that this reduced incidence of breast cancer is accompanied in each case by the appearance of significant levels of AFP in the rat sera; (Aim 2) to show that administering a hormone of pregnancy to SCID mice bearing human breast cancer xenografts would elicit murine AFP into the serum which would inhibit the growth of tumor xenograft, and that blocking AFP would in turn block the inhibition of breast cancer xenograft growth; and (Aim 3) to show that in hormone-treated mice, these responses occurring at the protein level can be demonstrated as well at the genome level, by quantifying the induction of mRNA for AFP in mouse liver.

BODY

Results

Specific Aim 1:

Five groups of carcinogen-exposed rats were treated with different hormones of pregnancy that has been previously shown [3-6] to protect against the induction of mammary cancer in rats, while a sixth group received no additional treatments. The outcomes in terms of breast cancer appearance are shown in Figure 1, Panels A-E. In each case, the upper curve shows cancer appearance in the positive control rats (animals that received only carcinogen) whereas the lower curve represents cancer appearance in animals treated with either E3 (Panel A); hCG (Panel B); E2 + P4 (Panel C); E3 + P4 (Panel D); or pregnancy (Panel E). In each case, the reduction of breast cancer incidence is similar to that reported by earlier workers (3-6). Table 1 indicates that the decreased incidence is statistically significant for each treatment (compared to control), that multiplicity was decreased by each treatment, and that tumor volume was decreased by each of these treatments.

Inhibition of Mammary Tumors in rats

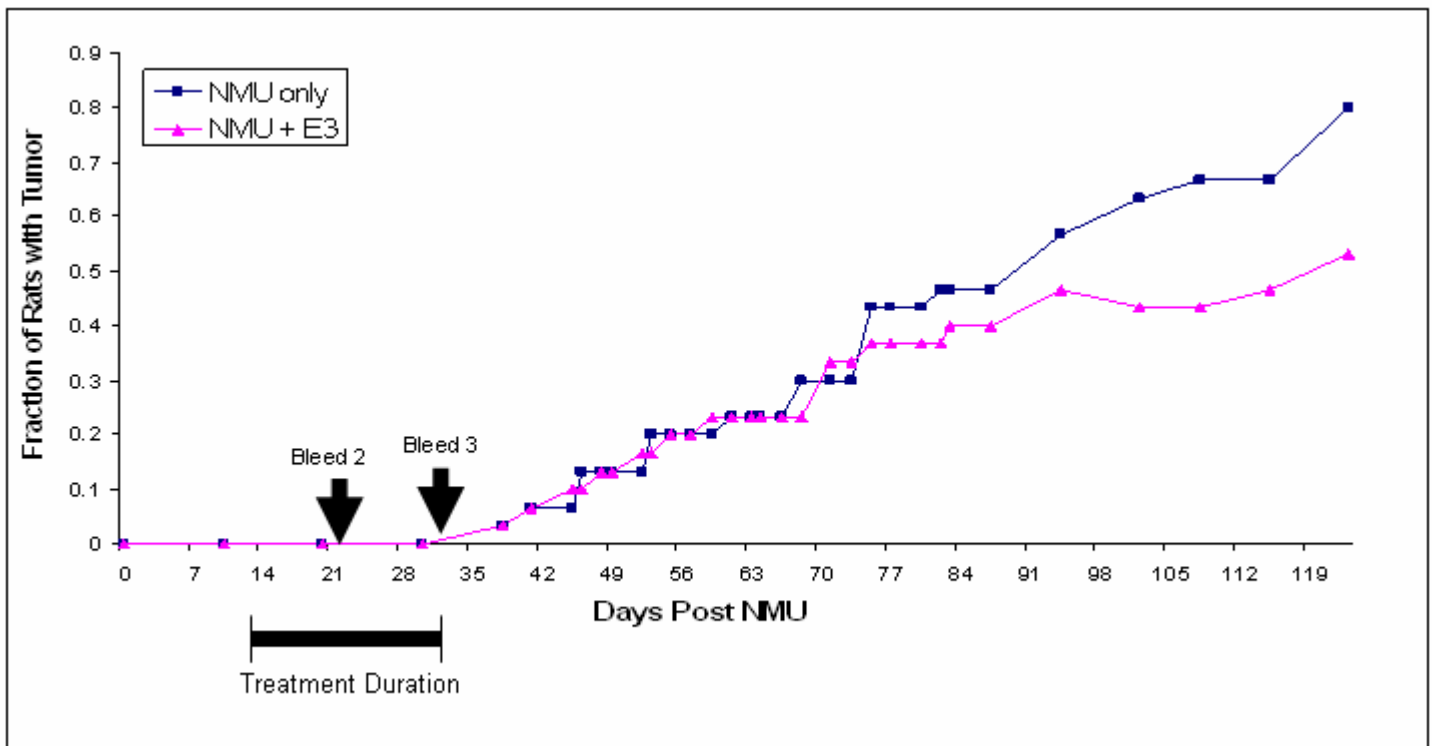


Figure 1a. Breast Cancer Incidence in Estriol (E3) Treated Sprague Dawley rats. Thirty Sprague-Dawley female virgin rats received NMU at the age of 50 days. After thirteen days, rats received a single subcutaneous silastic implant (0.078 inch IDx 0.125 inch OD, 2cm long) containing 30mg estriol and left under the skin for 21 days. At Day 102, tumor incidence was decreased by 27% ($p < 0.026$).

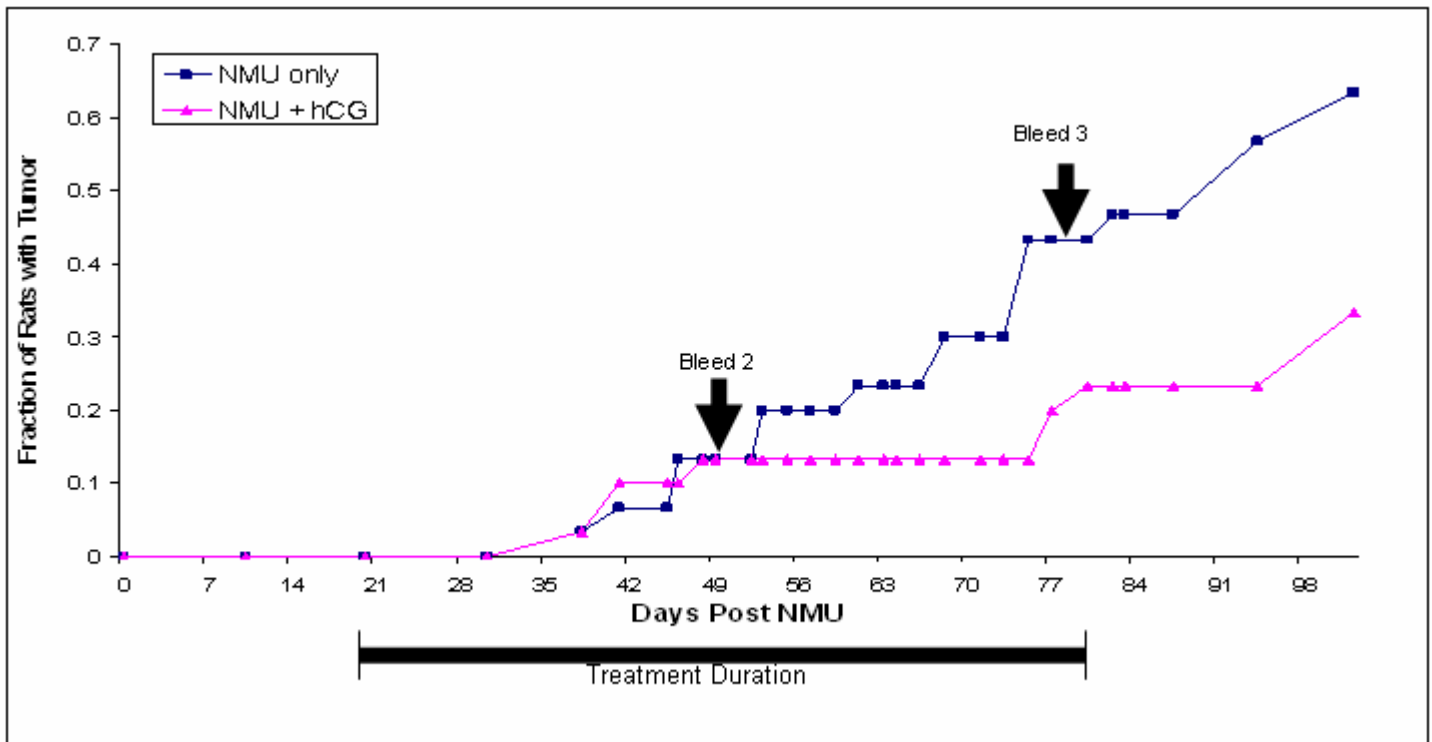


Figure 1b. Breast Cancer Incidence in Human Chorionic Gonadotropin (hCG) Treated Rats. Thirty Sprague-Dawley female virgin rats received NMU at the age of 50 days. After twenty-one days, rats were

administered an i.p. injection of 100 IU hCG daily, for 60 days. At Day 102, tumor incidence was decreased by 30% ($p < 0.02$).

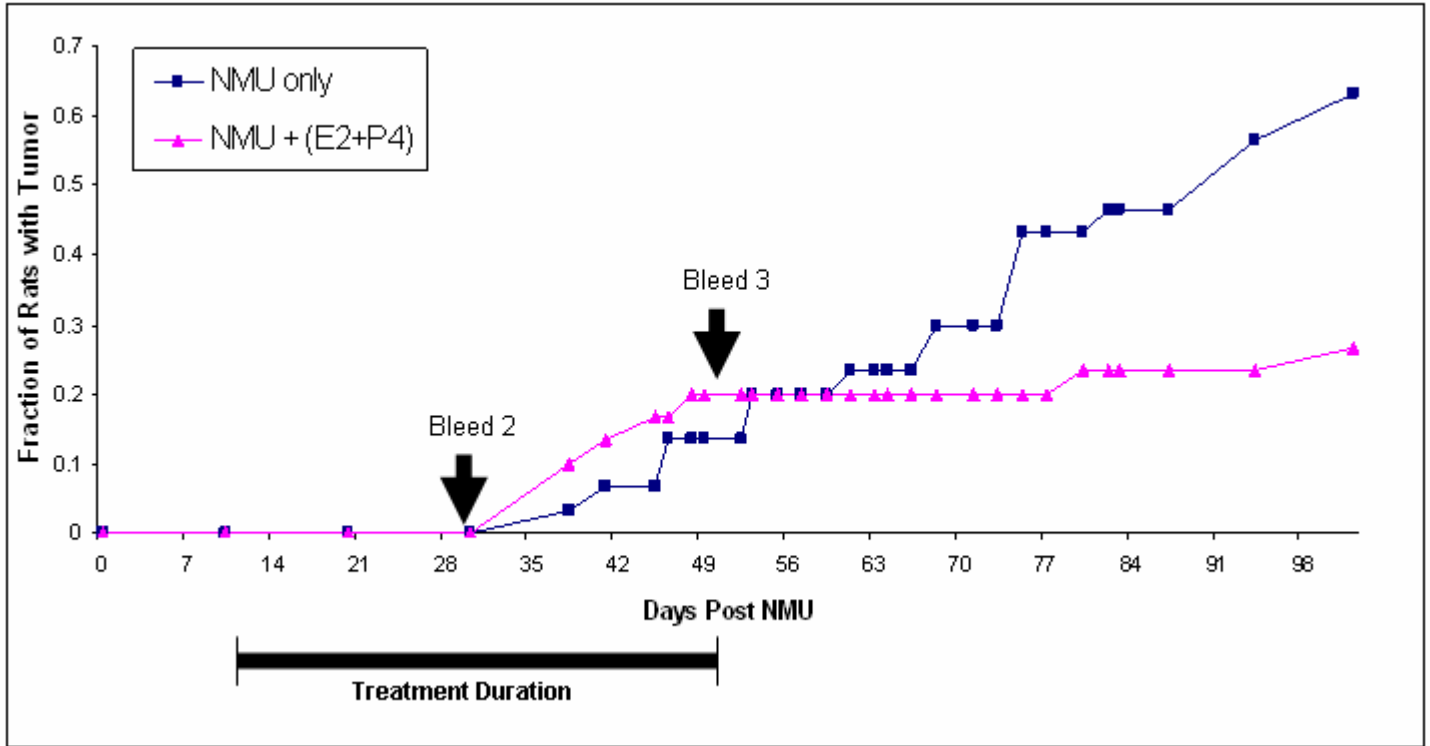


Figure 1c. Breast Cancer Incidence in Estrogen (E2) + Progesterone (P4) Treated Rats. Thirty Sprague-Dawley female virgin rats received NMU at age 50 days. After ten days, rats were administered a daily s.c. injection of 20µg E2 + 4mg P4, for 40 days. At Day 102, tumor incidence was decreased by 37% ($p < 0.0036$).

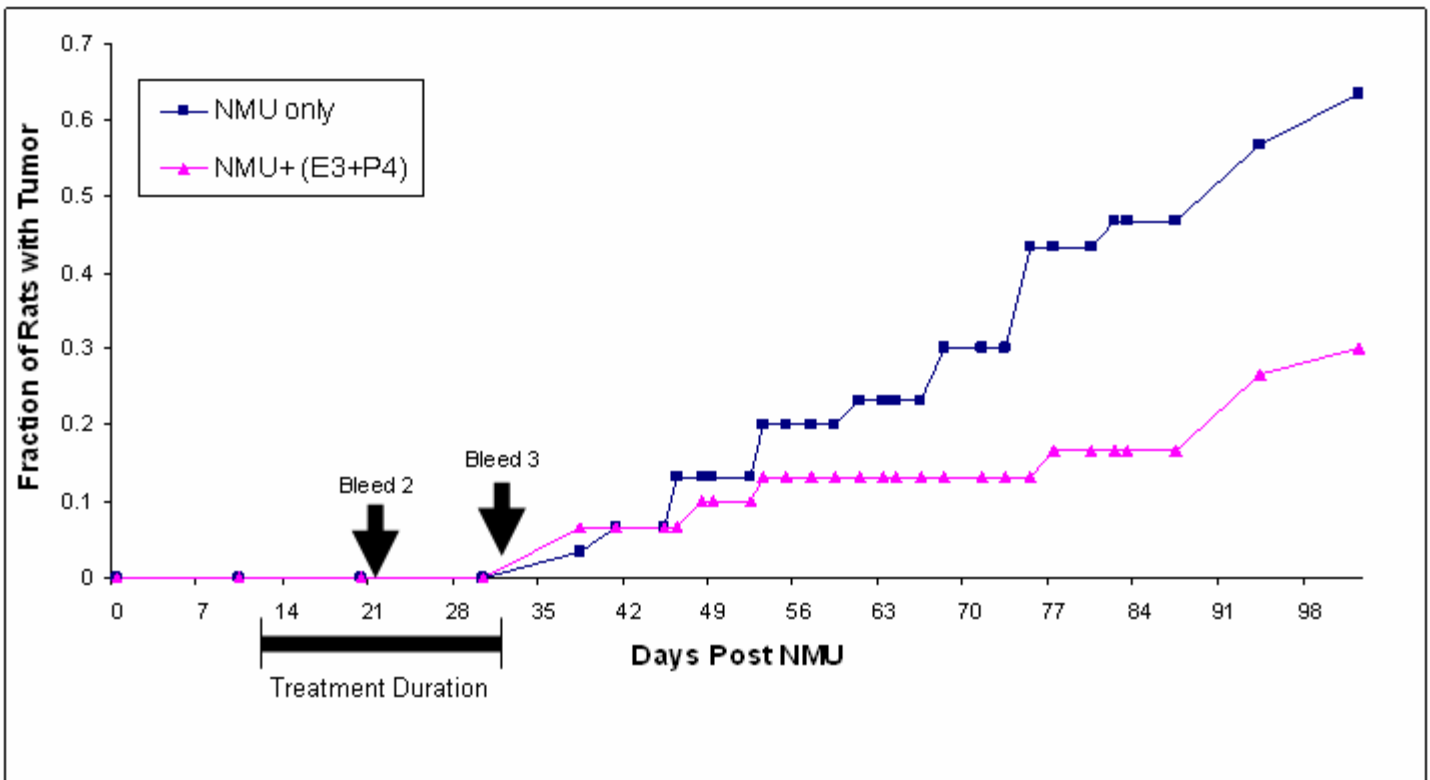


Figure 1d. Breast Cancer Incidence in Estriol (E3) + Progesterone (P4) Treated Rats. Thirty Sprague-Dawley female virgin rats received NMU at the age of 50 days. After thirteen days, rats received two subcutaneous silastic implants (0.078 inch IDx 0.125 inch OD, 2cm long), containing 30mg estriol and 30mg progesterone respectively, which were left in place for 21 days. At Day 102, tumor incidence was decreased by 33% ($p < 0.008$).

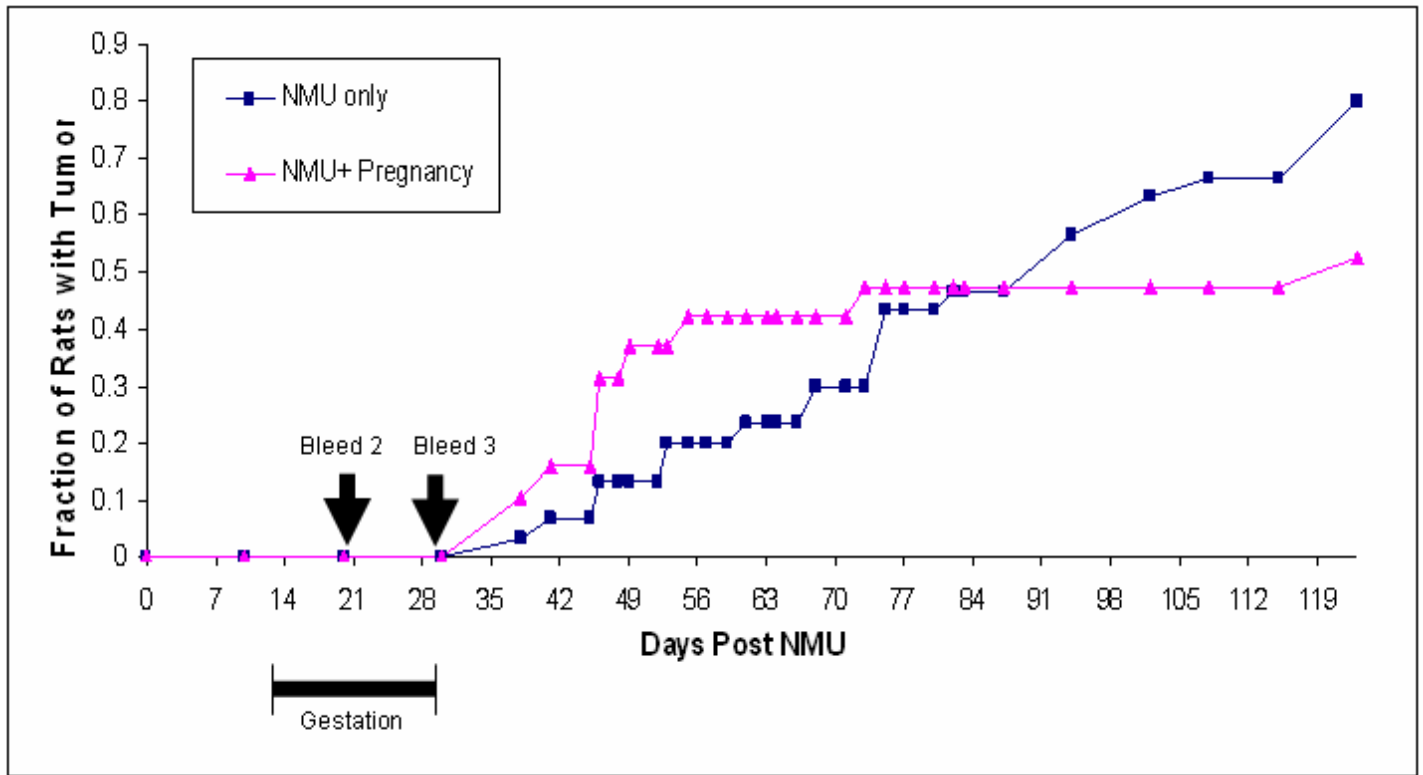


Figure 1e. Breast Cancer Incidence in Mated Female Rats. Thirty Sprague-Dawley female virgin rats received NMU at the age of 50 days (50mg/kg, intracarotid). After 10 days, 30 female rats were introduced to males (three females per male). Females stayed with males for 7 days, after which they were removed and separated. Twenty-one days later, 19 females bore litters, which were allowed to breast feed for 15 days. Females that did not become pregnant were excluded from the study. At Day 123, tumor incidence was decreased by 28% ($p < 0.035$). Note Crossover at Day 84.

Table 1. Treatment doses, schedules, and tumor incidence in NMU-treated Sprague Dawley female rats.

Treatment	No. of rats	Treatment Dose	Treatment Route	Treatment Start Date (Days Post MNU)	Treatment Duration (Days)	% Incidence (Day, p value) ¹	% Incidence (reference)	Tumors Per Group ²	Mean Number of Tumors Per Rat ²	Mean Tumor Volume ^{2,3} (mm ³)
Control	30	-	-	-	-	63.3 (102); 80.0 (123)		38	1.23	3504
E2 + P	30	20 µg E2 + 4 mg P	s.c. injection	10	40	26.6 (102, p < 0.0036)	20 (3)	10	0.33	2199
E3 + P	30	30 mg E3 + 30 mg P	Silastic Implant	13	21	30.0 (102, p < 0.008)	25 (4)	8	0.27	880
E3	30	30 mg E3	Silastic Implant	13	21	53.3 (123, p < 0.021) ⁶	64 (4)	24	0.8	955
hCG	30	100 IU hCG	i.p. injection	21	60	33.3 (102, p < 0.0014)	45 (6)	12	0.4	763
Pregnancy	19	-	-	10	21 ⁵	52.6 (123, p < 0.035) ⁶	45 (2)	28 ⁴	0.98	2649

¹ For difference from MNU control

² When calculated at Day 102

³ Sum of all tumor volumes in a group/total number of animals in group

⁴ Normalized for group size of 30 animals

⁵ Normal gestation period in rats

⁶ Incidence data collected at Day 123

Serum from animals in the prevention study was harvested at the midpoint of each regimen (Bleed 2) and again (from different animals) at the end of the treatment regimen (Bleed 3). Additionally, animals were bled one week after the end of the treatment regimen (Bleed 4). Rat AFP levels were estimated using Western blot analysis based on a standard curve using rat amniotic fluid AFP (Figure 2a). As shown in Figure 2b, each of the hormone treatment regimens resulted in an increase in serum AFP levels. Treatment with hCG elevated AFP levels 2.4-fold, E + P led to 1.9-fold elevation, E3 alone produced 2.9-fold elevation, and E3 + P produced a 3.2-fold rise in AFP levels.

Detection of Rat AFP by Western Blot AFP Standard Curve



Figure 2a. Western Blot of AFP Standard Curve. AFP levels were quantified in blood samples using a standard curve of amniotic fluid diluted in 1 x phosphate buffered saline (PBS). Amniotic fluid was drawn from the amniotic sac of 15 day pregnant Sprague Dawley female rats. Amniotic fluid was centrifuged three times at 2,500 rpm for five minutes to clarify. A western blot was run by diluting samples with 1 x PBS, loading 15ul of sample onto a 10% Tris HCl gel. Following electrophoresis protein was transferred to a PVDF membrane, blocked with a 5% dry milk solution, then blotted using a goat anti rat AFP HRP (1:200 in 5% milk TTBS) conjugated antibody (Santa Cruz Biological).

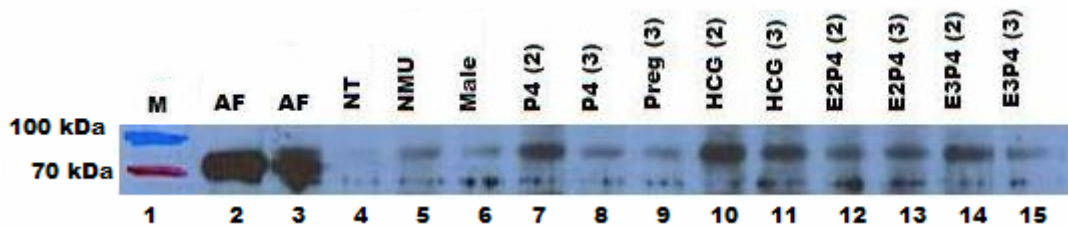


Figure 2b. Western blot detection of AFP in serum of hormone treated rats. NMU treated Sprague Dawley female rats subsequently treated with pregnancy-associated hormones were bled at the midpoint of hormone treatment (B2) and at the conclusion of hormone treatment (B3). Rat sera was separated from whole blood, AFP was isolated by immunoprecipitation (see Methods Section) and subsequent samples were run on a gel (Lanes 9-15) alongside an amniotic fluid standard (Lane 2, diluted 1:1000 in 1 x Phosphate Buffered Saline). Samples that underwent immunoprecipitation were not diluted (Lanes 3-15). Also included were a series of immunoprecipitated controls; amniotic fluid (Lane 3), untreated female rat sera (Lane 4), NMU treated female rat sera (Lane 5), and untreated male rat sera (Lane 6). Following electrophoresis protein was transferred to a PVDF membrane, blocked with a 5% dry milk solution, blotted overnight using a goat anti rat AFP (1:1000 in 5% milk TTBS), then with a rabbit anti goat IgG HRP (1:5000, Santa Cruz Biological).

Similar blots on sera were taken one week after the final hormone treatment (or parturition) and in each case significant AFP levels still were present (Figure 4). This indicates that AFP persists for at least 7 days following cessation of the hormonal manipulations, and may explain studies which have reported inhibition of breast cancer appearance even when the hormone is given before the carcinogen.

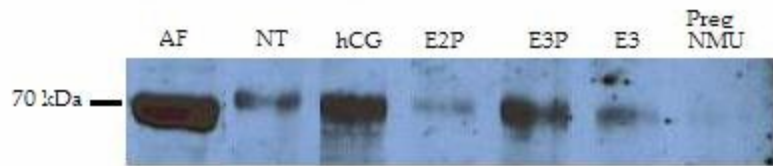


Figure 2c. Western Blots: AF in sera of NMU and hormone treated rats, at 7 days after hormone treatment ends.

Specific Aim 2:

The aim is to show that growth of human breast cancer xenografts growing in estrogenized immunodeficient mice is inhibited or blocked by a hormone of pregnancy (hCG) and that injecting the animals with hCG would elicit AFP from the adult liver. Further, mAFP would be found in sera of such mice. Additionally, in mice that have been passively immunized against mAFP, inhibition of tumor growth would not occur. This would demonstrate that the inhibition of human breast cancer xenograft growth was caused by its exposure to serum-born mAFP and not to some other substance elicited by the injection of hCG.

Severe Combined Immunodeficient (SCID) mice were divided into two groups, one designated to receive hCG and the other designated to receive vehicle for the duration of the experiment. hCG was given intraperitoneally once a day at a dose of 100 international units per mouse per day. This protocol was adopted from a report by Russo *et al.* (6), in which hCG had been shown to protect rats against development of DMBA-induced mammary tumors and verified by us that hCG protected rats against NMU-induced mammary tumors and induced AFP into blood. As shown in Figure 3, SCID mice were treated with hCG for 17 days prior to tumor implantation, a duration that was sufficient for induction of AFP into rat blood. For tumor implantation, human MCF-7 tumor xenografts growing in the mammary fat pad of donor SCID mice were harvested and cut into small pieces. Tumor pieces were carefully selected for homogeneous viable tumor, and transplanted under the kidney capsule of the vehicle-treated and hCG-treated SCID mice according to procedures previously published by our group (1). The subrenal capsule site was selected over the mammary fat pad or subcutaneous site because of the rapid and uniform rate of tumor growth in the subrenal capsule site (1). Tumor recipient mice also received subcutaneously an estradiol implant that provided a steady state level of 10^{-9} M estradiol in the blood which was needed to support growth of this tumor (1). Treatment with vehicle or hCG were continued daily after tumor implantation. Tumor size was measured during survival laparotomy at the time of tumor implantation, 14 days after tumor implantation and at necropsy 24 days after tumor implantation. Blood samples were obtained prior to hCG treatment, at the time of tumor implantation, and at necropsy. As shown in Figure 3, treatment with hCG significantly inhibited tumor growth. In the vehicle treated group tumor volume increased almost 3-fold (180%), (Figure 3a), whereas in the hCG-treated group tumor volume increased by only 33%, (Figure 3b). Thus in this experiment the overall inhibition of tumor growth by hCG was 60% (Figure 3c).

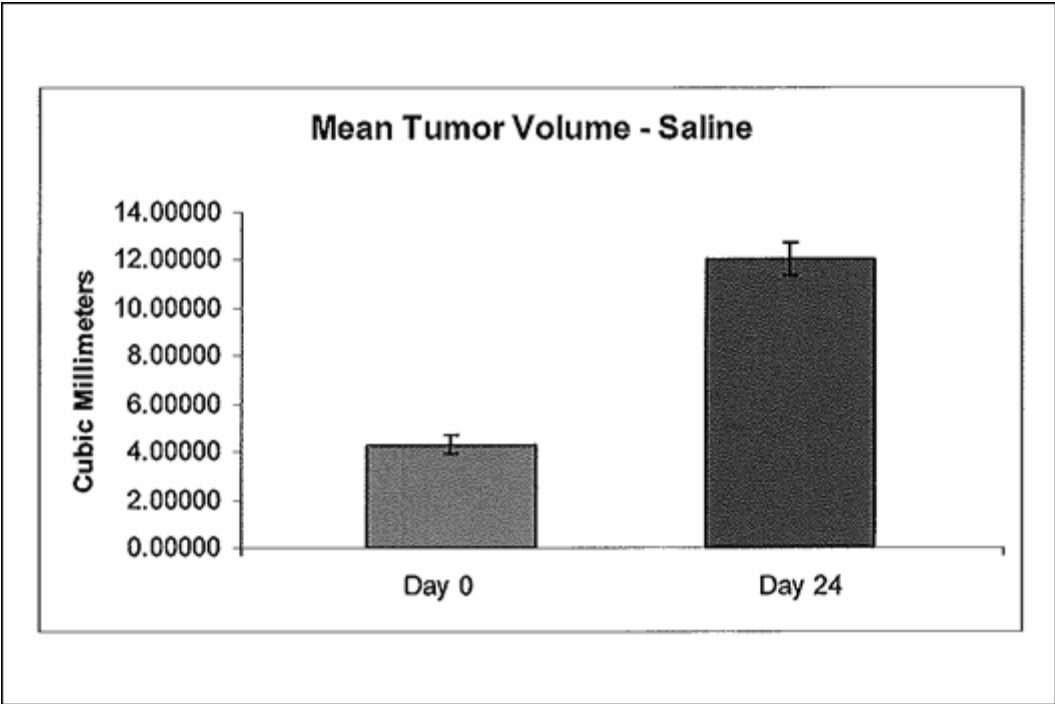


Figure 3a.

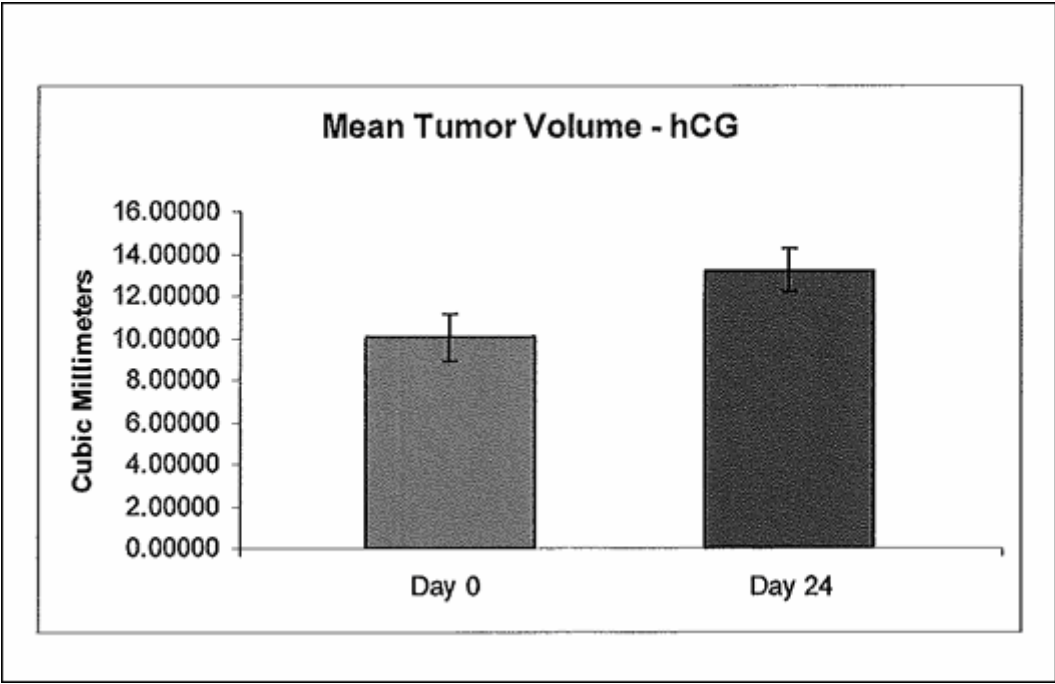


Figure 3b.

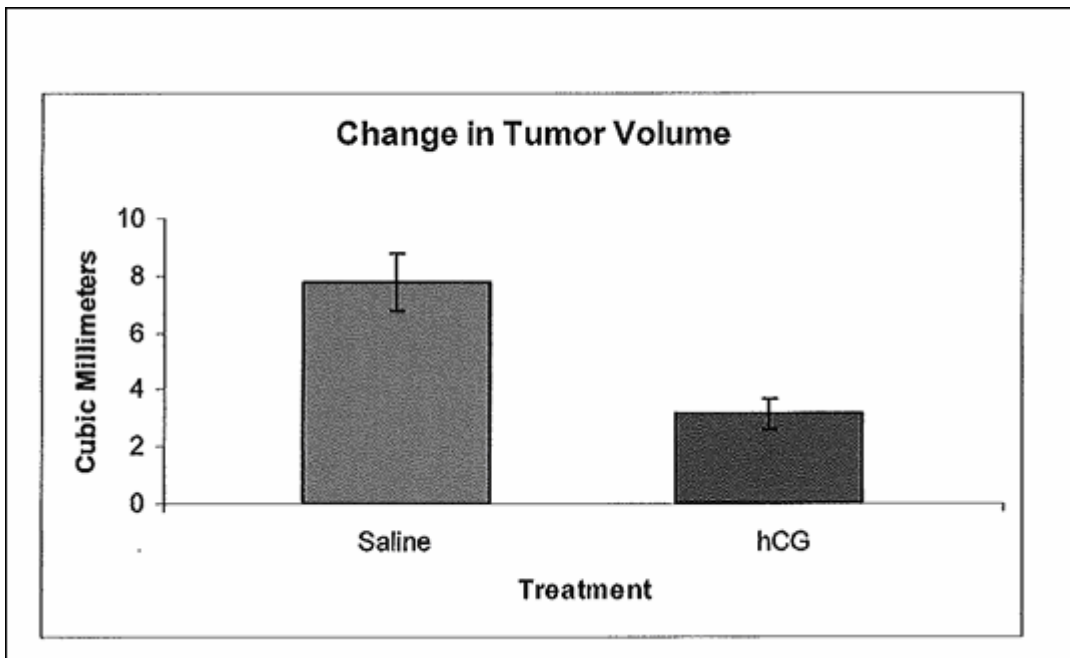


Figure 3c.

Specific Aim 2b:

Western blot analysis of the mouse sera provided no evidence that hCG administration had elicited murine AFP from these animals, even though the hCG treatment had demonstrably inhibited growth of the breast cancer xenografts that they bore – an anticipated effect of AFP. We suspected that this apparent disconnect was due to the nature of the AFP antigen contained in the serum. Rodent AFP consists of a mixture of as many as 8 isoforms that differ primarily in the nature and burden of their glycosylation. We tried multiple anti-AFP antibodies in multiple assay combinations and could not detect AFP in the sera from SCID mice that were treated with hCG. We conclude that the apparent absence of mAFP in the hCG treated SCID mice reflects the inability of our antibody to react with that substance. Clearly, using our available anti-AFP antibodies we could not achieve passive immunization of mice against mAFP, the experimental step originally planned.

We pursued the objective of Specific Aim 2 by an alternate route, which would involve the use of an all-human *in vitro* system consisting of human hepatoma cells (HepG2) which are known to secrete AFP, a potent antibody to human AFP, and human MCF-7 breast cancer cells.

As part of this Aim, we would also determine whether these hormones of pregnancy could also stimulate the human liver to produce AFP. Thus HepG2 cells were exposed to the pregnancy hormones (10^{-8} M for steroids and 10^{-9} M for hCG). Human AFP secreted into the medium was quantified by ELISA. The results show that hormone treatments that reduce the breast cancer incidence in rats also stimulate AFP production by HepG2 cells above the levels produced in untreated controls (Figure 4). On the other hand, treatment with P alone, which does not reduce the risk of breast cancer, also does not stimulate AFP production by HepG2 cells. These results support the hypothesis that hormones of pregnancy induce production of AFP by the liver and are consistent with the idea that AFP is the proximal protective agent that yields a reduced incidence of breast cancer in women who have experienced pregnancy.

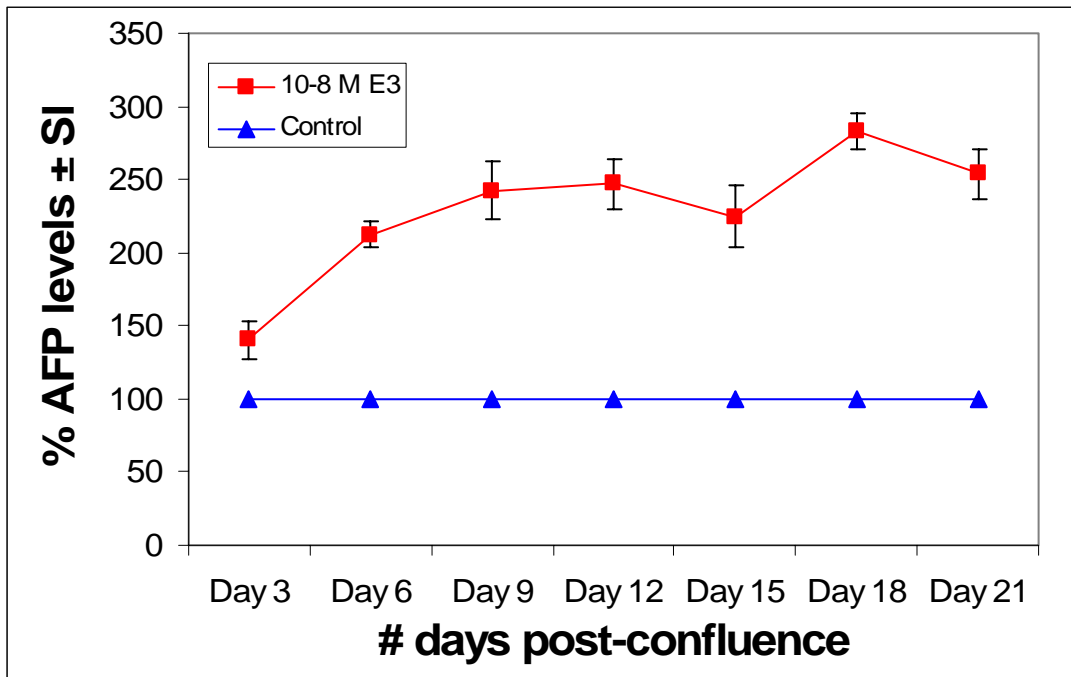


Figure 4a. AFP levels from HepG2 cells treated with 10-8M estriol (E3). 0.1×10^6 HepG2 cells were plated per well in a 24-well cell culture dish. After confluence was achieved, hormone treatment was started. The culture medium was extracted every 3 days and the concentration of AFP was determined by ELISA.

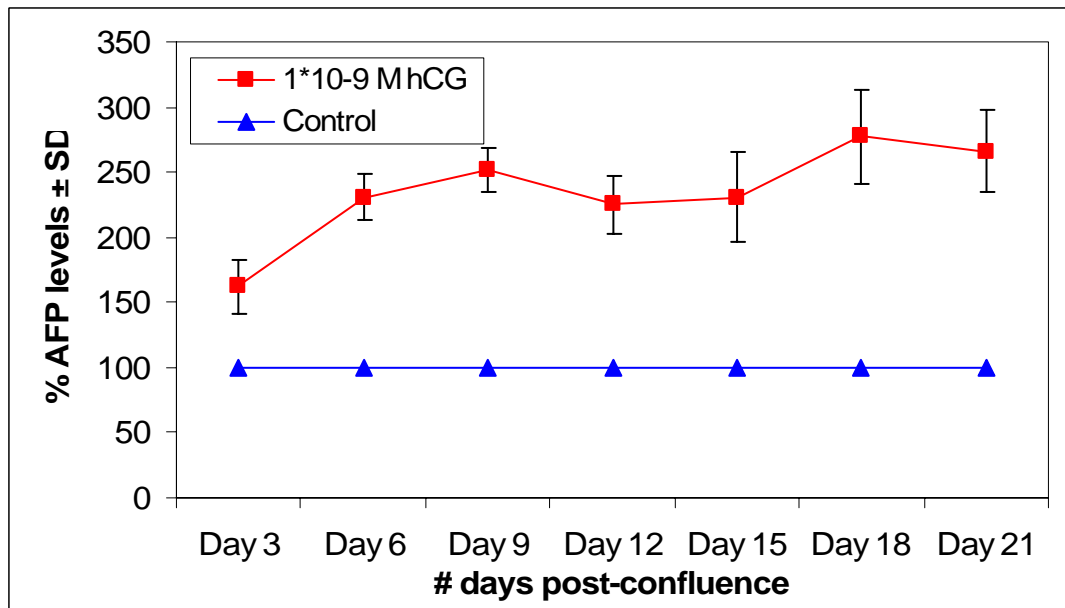


Figure 4b. AFP levels from HepG2 cells treated with 10-9M human chorionic gonadotropin (hCG). 0.1×10^6 HepG2 cells were plated per well in a 24-well cell culture dish. Confluence was achieved on Day 0 when hormone treatment was started. The culture medium was extracted every 3 days and the concentration of AFP was determined by ELISA.

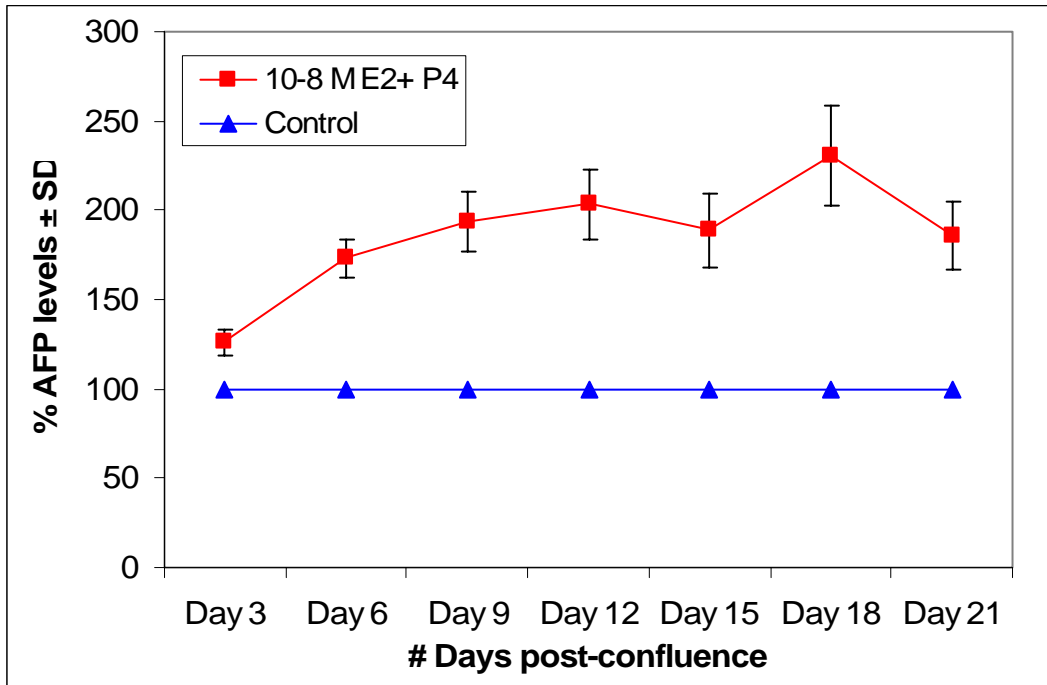


Figure 4c. AFP levels from HepG2 cells treated with 10-8M estradiol (E2) + 10-8M progesterone (P). 0.1*10⁶ HepG2 cells were plated per well in a 24-well cell culture dish. Confluence was achieved on Day 0 when hormone treatment was started. The culture medium was extracted every 3 days and the amount of AFP quantified by ELISA.

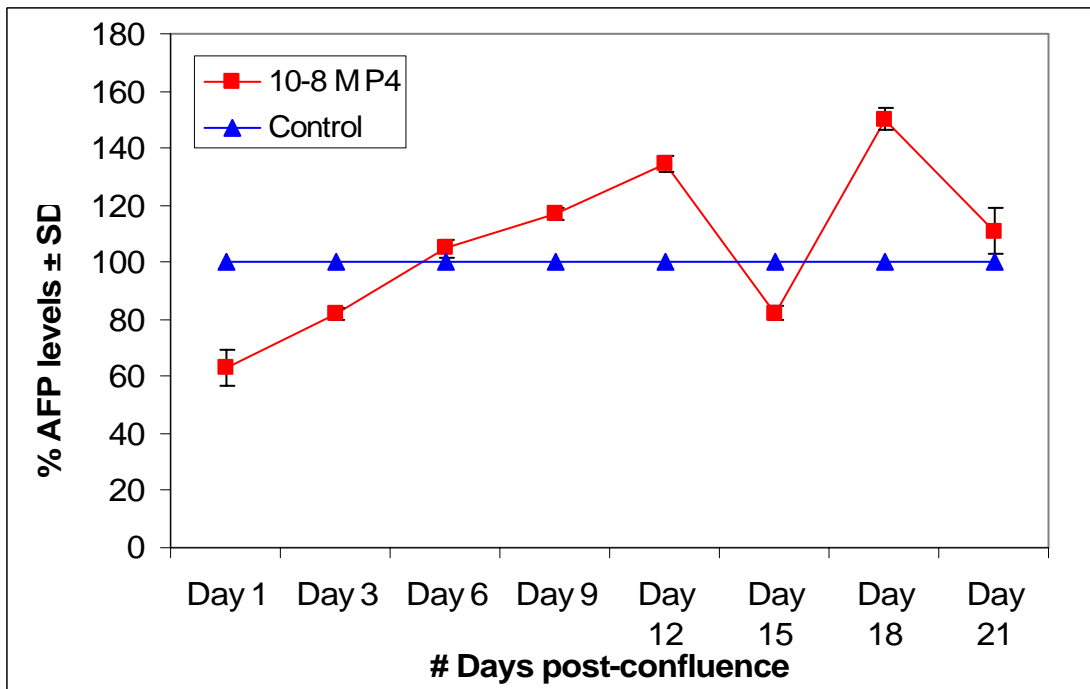


Figure 4d. AFP levels from HepG2 cells treated with 10-8M progesterone (P). 0.1*10⁶ HepG2 cells were plated per well in a 24-well cell culture dish. Confluence was achieved on Day 0 when hormone treatment was started. The culture medium was extracted every 3 days and the concentration of AFP was determined by ELISA.

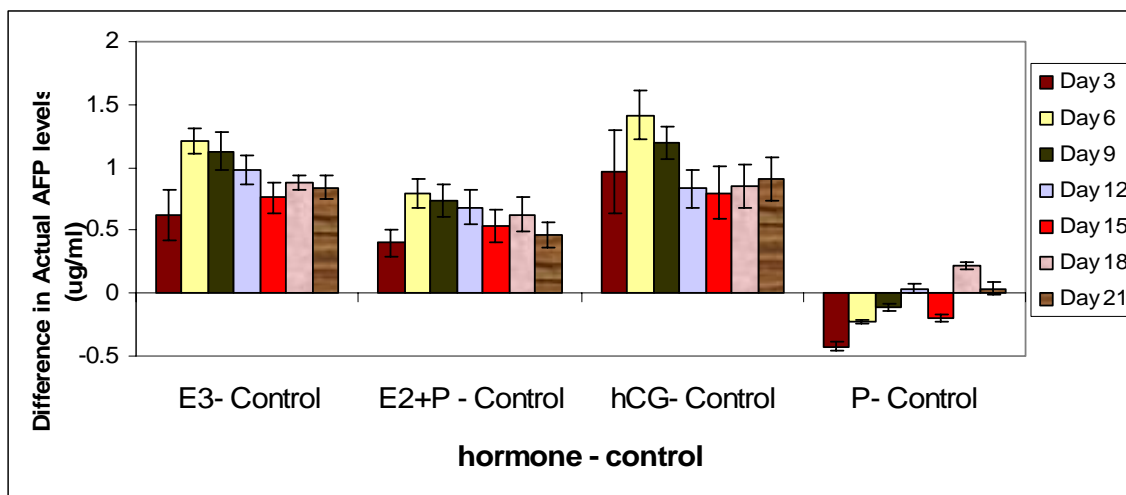


Figure 4e. Difference in AFP levels between hormone treated (lowest concentrations) and control groups. 0.1×10^6 HepG2 cells were plated per well in a 24-well cell culture dish. The medium was extracted every 3 days and the concentration of AFP was determined by ELISA. All three hormone treatments (especially at the lowest concentration) stimulate AFP production in HepG2 cells above levels in the controls.

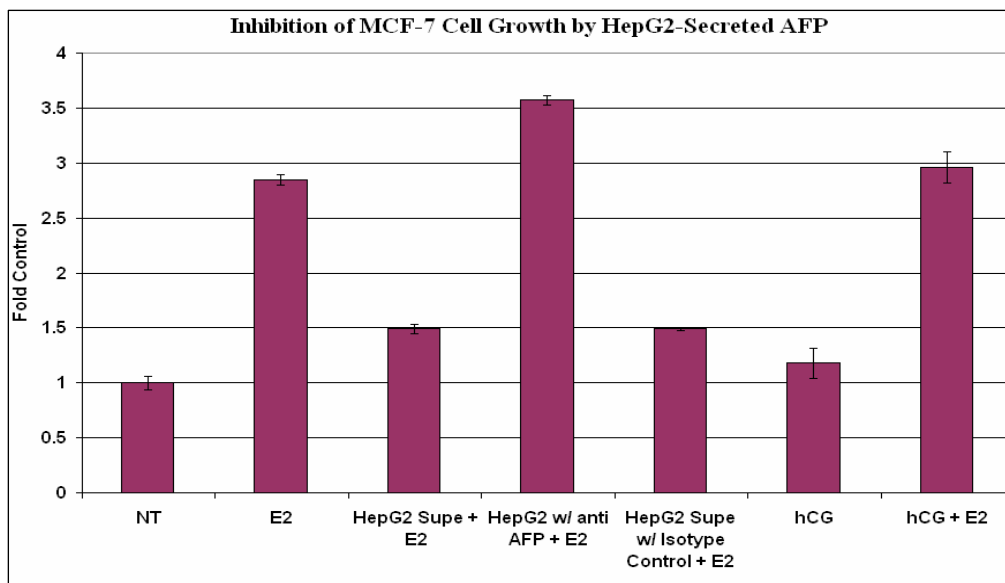


Figure 5.

In the studies shown in Figure 5, AFP enriched supernatant from HepG2 cells, that had been treated with hCG, inhibited the E_2 -stimulated growth of MCF-7 human breast cancer cells in culture. Antibody to AFP blocked the breast cancer growth inhibition by the AFP-containing supernatant. hCG alone (10^{-9} M) used to elicit AFP from HepG2 cells did not affect E_2 -stimulated growth of MCF-7 cells. We conclude that the principles proposed in Specific Aim 2 have been demonstrated; i.e., pregnancy hormone elicits AFP from the liver and liver-elicited AFP inhibits breast cancer growth.

Specific Aim 3:

RNA analyses. RNA extraction, Northern blot preparation and hybridization were carried out using conventional methods. Briefly, RNA was extracted from livers of control (C) or hCG (h) using Trizol according to the manufacturer (Invitrogen, Carlsbad, CA) and electrophoresed in a 1.0% agarose/formaldehyde gel. After transfer to Gene Screen (NEN Research Products, Boston, Mass), the RNA was fixed to the membrane with a UV Stratalinker 1800 (Stratagene Cloning Systems, La Jolla, CA). Blots were then prehybridized for 2.5 hours,

then hybridized overnight in 50 mM sodium phosphate buffer (pH 6.8), 1X SET, 5X Denhardt's, 0.5% SDS, and 200 µg/ml denatured, fragmented salmon sperm DNA at 65°C in a Hybaid hybridization oven (Laboratory Product Sales, Rochester, N.Y.). The hybridization solution contained random primed (Megaprime oligolabeling kit, Pharmacia, Piscataway, New Jersey) radiolabeled human AFP cDNA (OriGene, Rockville, MD) probe added to fresh prehybridization solution. After overnight hybridization, the blots were washed twice for a total of 40 minutes at 65°C with 2X SSC plus 0.2% SDS followed by 2 more washes for a total of 40 minutes at 65°C with 0.67X SSC plus 0.2% SDS. The blot signals were then captured by phosphoimaging.

The mouse liver studies show very little, if any, signal for AFP mRNA. This may be due to the use of a human cDNA probe. However, only moderate blot washing stringency was used and should have allowed for hybridization of the human probe to the mouse liver mRNA. Thus, the more likely explanation is that there is minimal expression of liver AFP mRNA, and that this is at or below the level of Northern blot sensitivity.

In contrast, human HepG2 cells showed significant levels of AFP mRNA. However, the HepG2 studies revealed that there were no significant changes in AFP mRNA levels in HepG2 cells treated for various times with hCG (h) (Figure 6). This suggests that the observed increase in AFP protein (Figure 4) is not due to a corresponding increase in AFP mRNA levels (Figure 6), but rather due to some post-transcriptional regulation such as differential AFP protein stability.

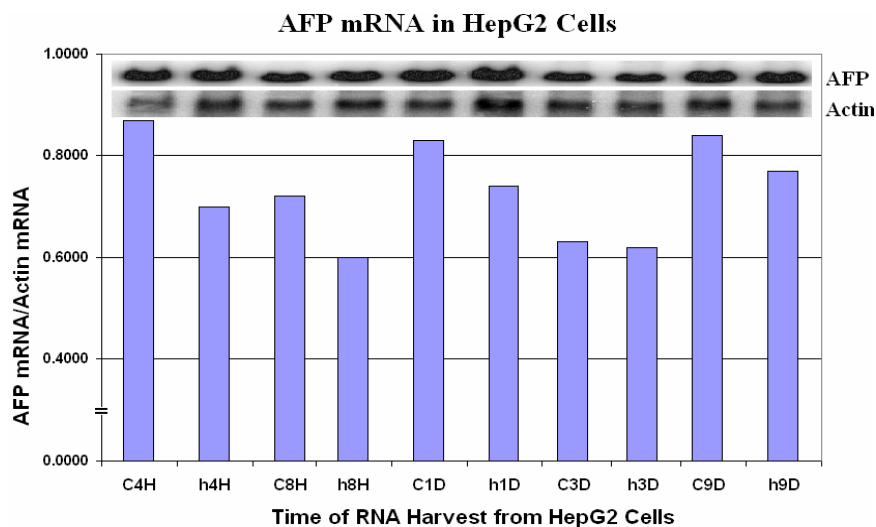


Figure 6.

KEY RESEARCH ACCOMPLISHMENTS

1. The hormones of pregnancy reduce the incidence of NMU-induced mammary cancer in rats.
2. The hormones of pregnancy increase the level of AFP in rat blood.
3. The hormones of pregnancy elicit the secretion of AFP from cultured human liver cells. The increase in AFP is a post transcriptional event. The AFP-containing culture medium inhibits the growth of human breast cancer cells. Antibody to AFP neutralizes the growth inhibitory effect of that culture medium.

REPORTABLE OUTCOMES

Posters

1. Jacobson HI, Andersen TT, Bennett JA, Lemanski N. "Exploiting for Breast Cancer Control a Proposed Unified Mechanism for Reduction of Human Breast Cancer Risk by the Hormones of Pregnancy". U.S. Department of Defense Era of Hope Meeting, Philadelphia, 2005.
2. Jacobson HI, Andersen TT, Bennett JA, Lemanski N. "Exploiting for Breast Cancer Control a Proposed Unified Mechanism for Reduction of Human Breast Cancer Risk by the Hormones of Pregnancy". American Association for Cancer Research, Frontiers in Cancer Prevention Meeting, Baltimore, 2005.
3. Jacobson HI, Lemanski N, Narendran A, Agarwal A, Bennett JA, Andersen T. "Hormones of Pregnancy, AFP, and Reduction of Breast Cancer Risk." Fifth International Conference on Hormonal Carcinogenesis, Montpellier France, 2006.
4. Jacobson HI, Lemanski N, Narendran A, Agarwal A, Crawford D, Bennett JA, Andersen TT. "Hormones of Pregnancy, AFP, and Reduction of Breast Cancer Risk." U.S. Department of Defense Era of Hope Meeting, 2008.

Publication

1. Jacobson HI, Lemanski N, Narendran A, Agarwal A, Bennett JA, Andersen TT. "Hormones of Pregnancy, AFP and Reduction of Breast Cancer Risk." In: Hormonal Carcinogenesis V, Chapter 34. JJ Li and S A Li eds., Springer Verlag, 2007.

Manuscript

1. Jacobson HI, Lemanski N, Narendran A, Agarwal A, Bennett JA, Andersen TT. "Hormones of Pregnancy, AFP and Reduction of Breast Cancer Risk." Prepared for submission to Proceedings of the National Academy of Sciences of the U.S. by an Academy member.

Presentations

1. AFP, Parity and Cancer. CORE Curriculum, Department of Pathology, Albany Medical College.
2. How Parity Affects Breast Cancer Risk. University of Cincinnati Medical School, 2006.

CONCLUSION

1. The hypothesis we have proposed is supported by our findings. The ability of several different hormones of pregnancy (E2, E3, P4 or hCG) to inhibit breast cancer appearance in carcinogen-treated rats reflects that these agents elicit hepatic secretion of AFP, which is then the proximal antioncotic agent.
2. The secretion of AFP by cultured HepG2 liver cancer cells when stimulated by treatment with hCG occurs without increased cellular AFP mRNA content. This suggests that the stimulation is effected at a post translation site, possibly operating on mRNA and/or protein stability.

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